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Localization of an α-Amanitin Resistance Mutation in the Gene Encoding the Largest Subunit of Mouse RNA Polymerase II

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RNA polymerase II is inhibited by the mushroom toxin α-amanitin. A mouse BALB/c 3T3 cell line was selected for resistance to α-amanitin and characterized in detail. This cell line, designated A21, was heterozygous, possessing both amanitin-sensitive and -resistant forms of RNA polymerase II; the mutant form was 500 times more resistant to α-amanitin than the sensitive form. By using the wild-type mouse RNA polymerase II largest subunit (*RPII215*) gene (J. A. Ahearn, M. S. Bartolomei, M. L. West, and J. L. Corden, submitted for publication) as the probe, *RPII215* genes were isolated from an A21 genomic DNA library. The mutant allele was identified by its ability to transfer amanitin resistance in a transfection assay. Genomic reconstructions between mutant and wild-type alleles localized the mutation to a 450-base-pair fragment that included parts of exons 14 and 15. This fragment was sequenced and compared with the wild-type sequence; a single AT-to-GC transition was detected at nucleotide 6819, corresponding to an asparagine-to-aspartate substitution at amino acid 793 of the predicted protein sequence. Knowledge of the position of the A21 mutation should facilitate the study of the mechanism of α-amanitin resistance. Furthermore, the A21 gene will be useful for studying the phenotype of site-directed mutations in the *RPII215* gene.

RNA polymerase II is a multisubunit enzyme that transcribes protein-coding genes in eucaryotes (see references 10, 34, and 45 for reviews). Although little is known about the functional role of individual subunits, the analysis of RNA polymerase II has been facilitated by use of the mushroom toxin α-amanitin. At low concentrations, α-amanitin selectively inhibits RNA polymerase II from most species (32, 33, 35). α-Amanitin binds to RNA polymerase II with an equilibrium association constant of approximately 10^{10} M^{-1} and inhibits transcription by blocking elongation of the enzyme after the formation of a phosphodiester bond (13, 54). The binding of a single toxin molecule to RNA polymerase II is sufficient to inhibit transcription (13).

Mutations that give rise to an α-amanitin-resistant phenotype have been described for cell lines from several species (7, 11, 29, 50, 51). In these cell lines, the mutation conferring α-amanitin resistance renders the enzyme 5 to 1,000 times more resistant to α-amanitin. Hybrids of Chinese hamster ovary (CHO) cells that were constructed from α-amanitin-sensitive and -resistant cells express both RNA polymerase II alleles (36). However, when these hybrid lines are grown in the presence of α-amanitin, only the α-amanitin-resistant enzyme is expressed (26). In this case, there appears to be enhanced degradation of the α-amanitin-sensitive protein together with a compensatory increase in the synthesis of the α-amanitin-resistant protein (27). Codominance of RNA polymerase II expression has also been observed in α-amanitin-resistant L6 rat myoblast cells (17).

α-Amanitin resistant *Drosophila melanogaster* (23) and *Caenorhabditis elegans* (46) strains have also been isolated. The mutation in the α-amanitin-resistant *Drosophila* mutant C4 was mapped to band 10C on the X chromosome (24), and this region was subsequently found to contain a structural gene for an RNA polymerase II subunit (15). By using P element insertional mutagenesis, a lethal mutation was introduced into the *C4* locus. The DNA surrounding the insertion was isolated by using P element DNA as a probe (49) and was later found to encode the largest subunit (*RPII215*) of RNA polymerase II (3, 22). The *RPII215* gene from *Drosophila* has been used to isolate the analogous genes from *Saccharomyces cerevisiae* (30), human (12), and mouse DNA (J. A. Ahearn, M. S. Bartolomei, M. L. West, and J. L. Corden, submitted for publication).

Amanitin resistance mutations are of interest not only from the standpoint of understanding the mechanism of inhibition by the toxin but also because amanitin-resistant organisms (24) and cell lines (18, 42) show developmental defects, possibly due to altered promoter selectivity of the mutant polymerase. The study of amanitin-resistant RNA polymerase may therefore be useful in elucidating the mechanism of transcriptional control of protein-coding genes. A prerequisite for this study is a well-characterized α-amanitin resistance gene.

In this paper we report the isolation and localization of an α-amanitin resistance mutation in the mouse *RPII215* gene. We first isolated α-amanitin-resistant mouse BALB/c 3T3 cell lines after ethyl methanesulfonate mutagenesis. *RPII215* genes from one cell line, A21, were isolated by using a probe from the wild-type *RPII215* gene (Ahearn et al., submitted). The mutant allele was identified by DNA-mediated transfer of amanitin resistance, and genomic reconstruction experiments were used to localize the α-amanitin resistance mutation to a 450-base-pair restriction fragment. Both strands of this fragment were sequenced, and comparison of the mutant sequence with the wild-type sequence showed a single AT-to-GC change at nucleotide 6819, corresponding to an asparagine-to-aspartate change at amino acid 793 of the predicted protein sequence. The potential applications of this mutant gene are discussed.

MATERIALS AND METHODS

Cell lines. BALB/c 3T3 cells (American Type Culture Collection, Rockville, Md.) were used as the parent line for the selection of α-amanitin-resistant cell lines. Cells were

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grown as monolayer cultures in minimal essential medium plus 10% fetal bovine serum (MEM-10) and were sensitive to α-amanitin (3 μg/ml) (Boehringer Mannheim, Indianapolis, Ind.) and G418 (400 μg/ml) (Gibco Laboratories, Grand Island, N.Y.). All α-amanitin-resistant cell lines were grown in the presence of 3 μg of α-amanitin per ml. L6 rat myoblasts (provided by Mark Pearson, Du Pont, Wilmington, Del.) and thymidine kinase-deficient mouse L cells (Ltk^-; provided by Jordan Kreidberg, Johns Hopkins University School of Medicine) were grown as monolayer cultures in Dulbecco modified Eagle medium plus 10% fetal bovine serum. Both lines were sensitive to α-amanitin (2 μg/ml) and G418 (300 μg/ml).

Selection of mutants. BALB/c 3T3 cells were plated in 75-cm^2 flasks (Falcon Plastics, Oxnard, Calif.). When the cells were 75% confluent, the old medium was removed and replaced with MEM-10 plus ethyl methanesulfonate (300 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). After 20 h the medium was removed, the cells were washed once with phosphate-buffered saline, and fresh MEM-10 was added to the flask. Three days later the cells were split 1:2 and plated in MEM-10 plus α-amanitin (5 μg/ml). The medium was changed every 4 to 5 days throughout the selection. Surviving colonies were picked and plated in 24-well Costar dishes (Cambridge, Mass.) containing MEM-10 plus 5 μg of α-amanitin per ml.

RNA polymerase assays. For RNA polymerase assays, cells were either grown with α-amanitin or grown without α-amanitin at least 10 days prior to harvesting. Extracts were prepared from 5×10^6 cells as described by Somers et al. (51). Cells were trypsinized and suspended in 10 ml of MEM-10, pelleted by centrifugation (1,000 rpm for 5 min), and washed with phosphate-buffered saline containing phenylmethylsulfonyl fluoride (10^{-4} M). After centrifugation, cells were suspended in 0.5 ml of a solution containing 0.05 M Tris hydrochloride, pH 7.9, 25% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 10^{-4} M phenylmethylsulfonyl fluoride and lysed by four cycles of freezing in liquid nitrogen and thawing at 30°C to make them permeable to nucleoside triphosphates. A transcription mix containing [^{3}H]UTP was added to the lysed cells, and transcription by previously engaged polymerase was allowed to proceed. Incorporation of [^{3}H]UTP was measured by DEAE-cellulose filter binding (Whatman, DE81). Because of the presence of manganese ions and a high ammonium sulfate concentration (0.4 M) in the assay mix, this assay primarily detects RNA polymerase III activity.

Isolation of genomic clones from A21 cells. Two genomic DNA libraries were constructed from the cell line A21 in bacteriophage λL47 (38). High-molecular-weight DNA was prepared (25), and 1 mg was digested with *Eco*RI or *Hin*dIII. The DNA was size fractionated by preparative gel electrophoresis on a Hoefer bull's eye gel apparatus (San Francisco, Calif.). Fractions were collected at 2-h intervals, and 1/20 of each fraction was ethanol precipitated and size fractionated on a 1% agarose gel. The gel was transferred to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) as described by Southern (52), and the filters were hybridized to nick-translated (44) probe BE2.9 (see Fig. 2). Fractions containing the 18-kilobase (kb) *Hin*dIII and 14-kb *Eco*RI fragments were phenol and chloroform extracted and ethanol precipitated. Fractionated A21 DNA (430 ng) was ligated to *Eco*RI- or *Hin*dIII-digested λL47 DNA (1.35 μg) in a 10-μl reaction mixture (19). From these reactions, 1 μl was packaged into phage with Gigapack in vitro packaging extract (Vector Cloning Systems, San Diego, Calif.). Phage were absorbed to *Escherichia coli* (strain P2rk-mk^+) cells and plated on T agar plates (20 by 20 cm) containing 10 g of tryptone and 15 g of agar per liter; 100,000 phage were screened by hybridization to the BE2.9 probe by the procedure described by Benton and Davis (2). Positive phage were isolated by two rounds of hybridization screening and plaque purification. Phage lysates were prepared by the method of Blattner et al. (5), and phage were purified by the procedure of Yamamoto et al. (58). After cesium chloride banding, the phage were dialyzed against TE (10 mM Tris hydrochloride, pH 7.6, 1 mM EDTA), digested with 50 μg of proteinase K per ml, and phenol and chloroform extracted. The phage DNA was again dialyzed against TE.

Inserts from a few phage were isolated by digestion with *Eco*RI or *Hin*dIII and cloned into the *Eco*RI or *Hin*dIII sites of pKP58, a deletion mutant of pBR322 (K. Peden, unpublished data). pH19-4 and pH22-1 are plasmids with *Hin*dIII inserts derived from cell line A21. pE26-7 and pE26-4 are plasmids with *Eco*RI inserts derived from cell line A21. Plasmid pHRPII contains an *Hin*dIII insert derived from a mouse BALB/c 3T3 library (Ahearn et al., submitted). pEE14.3 and λGA_1 were derived from a BALB/c mouse embryo library (Ahearn et al., submitted). Plasmid pEXRPII3′ has the 3′ 11-kb *Xho*I-*Eco*RI fragment from pEE14.3 cloned into the *Eco*RI-*Xho*I region of pKP58. Plasmid DNA was prepared by the method of Birnboim and Doly (4) as modified by Ish-Horowicz and Burke (31). For large-scale (300 ml) preparations, plasmids were banded twice in cesium chloride-ethidium bromide gradients. After removal of the ethidium bromide with *tert*-isobutanol, DNA was precipitated with ethanol, dissolved in TE, and purified by proteinase K digestion, phenol extraction, and isopropanol precipitation.

Transfections. To prepare DNA for transfection, phage or plasmid DNA was digested with *Xho*I, phenol and chloroform extracted, and ethanol precipitated. Equal amounts of DNA from the 5′ *Hin*dIII and 3′ *Eco*RI clones were ligated, phenol and chloroform extracted, and ethanol precipitated. DNA was suspended in 0.1× TE at a concentration of 500 μg/ml.

L6 rat myoblasts, mouse BALB/c 3T3 cells, and mouse Ltk^- cells were plated on 6-cm dishes at approximately 5×10^4 cells per dish the day before transfection. Ten micrograms of DNA consisting of test DNA (see figure legends for exact amounts), 1 μg of pSV2neo (53), and salmon sperm DNA was introduced by the calcium phosphate procedure (56) and glycerol shock (20). One day later each dish was split into three dishes, and after 2 days selection with G418 or α-amanitin was started. The α-amanitin concentration was increased gradually from 0.5 to 2 or 3 μg/ml in three succesive feedings. Dishes were fed every 3 days, and after 2 to 3 weeks colonies were fixed with 10% formaldehyde and stained with Giemsa. A few colonies were isolated for further analysis.

DNA sequencing. Dideoxy sequencing was performed as described by Sanger et al. (48), and the products were resolved on 6 and 8% polyacrylamide gels (47).

RESULTS

Selection of α-amanitin-resistant BALB/c 3T3 cell line A21. BALB/c 3T3 cells were mutagenized with ethyl methanesulfonate, and cells were plated in the presence of α-amanitin (5 μg/ml). Mutant cell lines were isolated at a frequency of $\sim 10^{-6}$, or about 10 times above the background of spontaneous mutation. Surviving colonies were ex-

panded, and their RNA polymerase activities were assayed (51) to determine the level of resistance. Cell lysates were assayed in the presence of increasing concentrations of α-amanitin, and the activity was expressed as a fraction of the counts incorporated when no amanitin was present. Different cell lines possessed RNA polymerase II activities that were 10 to 1,000 times more resistant to α-amanitin than the wild-type enzyme. The polymerase assay for one mutant, A21, is shown in Fig. 1A. When cell line A21 was assayed after being cultured without α-amanitin for 10 days, both sensitive and resistant forms of RNA polymerase II were active. The biphasic curve shown in Fig. 1A resulted from these two activities. The curve could be resolved into two components: the first is representative of an enzyme with the inactivation pattern of wild-type RNA polymerase II, and the second corresponds to an enzyme inactivated at a 500-fold-higher α-amanitin concentration. Culturing A21 cells in the presence of α-amanitin prior to the assay resulted in transcription solely by the resistant form of the enzyme, as indicated by the monophasic curve generated in the assay (Fig. 1A). These results demonstrate that cell line A21 has two forms of RNA polymerase: an α-amanitin-sensitive wild-type form and an α-amanitin-resistant form. Both enzymes were expressed when cells were cultured without α-amanitin, but only the resistant enzyme was expressed when cells were cultured with α-amanitin.

Isolation of RPII215 genes from α-amanitin-resistant line A21. Because the mutation to α-amanitin resistance in the *Drosophila* C4 mutant line is in the *RPII215* subunit (22), it seemed likely that the mutation conferring α-amanitin resistance in A21 cells would be in the analogous mouse gene. To isolate the *RPII215* genes from line A21, a probe from the wild-type BALB/c mouse *RPII215* gene (Ahearn et al., submitted) was used. *Eco*RI and *Hin*dIII libraries were made by cloning genomic DNA from line A21 into bacteriophage λL47. The *RPII215* gene was cloned in two overlapping fragments (Fig. 2): an 18-kb *Hin*dIII fragment (5′ clones) and a 14-kb *Eco*RI fragment (3′ clones). The overlapping 3.3-kb region contained a unique *Xho*I site, which was used to recombine the two pieces of the gene, and also encompassed a unique 2.9-kb *Bam*HI-*Eco*RI fragment (BE2.9), which was used to screen libraries. A number of clones from each library were isolated to ensure that at least one clone from each allele was present.

Transfection assay for identifying the α-amanitin resistance allele. A transfection assay was developed to identify the mutant allele. DNA from individual BALB/c 3T3 and A21 5′ and 3′ clones was digested with *Xho*I and ligated to produce intact genes. L6 rat myoblasts (see below) were transfected with test DNA, and the transfer of α-amanitin resistance was assayed. To control for variations in the efficiency of transfection, pSV2neo DNA was cotransfected and transfer of G418 resistance was also assayed. When wild-type 5′ (pHRPII) and 3′ (pEXRPII3′) clones were ligated and transfected, no α-amanitin-resistant colonies were detected (Table 1). Similarly, ligated 5′ A21 clones (pH19-4 and pH22-1) and wild-type 3′ clones (λGA$_1$ and pEE14.3) transferred only background levels of resistance. However, when the 3′ clone pE26-7 from cell line A21 was ligated either to wild-type (pHRPII) or to A21 5′ clones (pH19-4 and pH22-1), α-amanitin-resistant colonies were detected. These results demonstrate that pE26-7 was derived from the mutant A21 allele and that the mutation causing α-amanitin resistance resided on the 3′ fragment of the *RPII215* gene. Since the reconstructed *RPII215* gene containing the 3′ clone pE26-4 from A21 did not transfer resistance, it probably was derived from the sensitive allele. The few resistant colonies found with ligated pH22-1 and pEE14.3 and with pH22-1 and pE26-4 were not characterized.

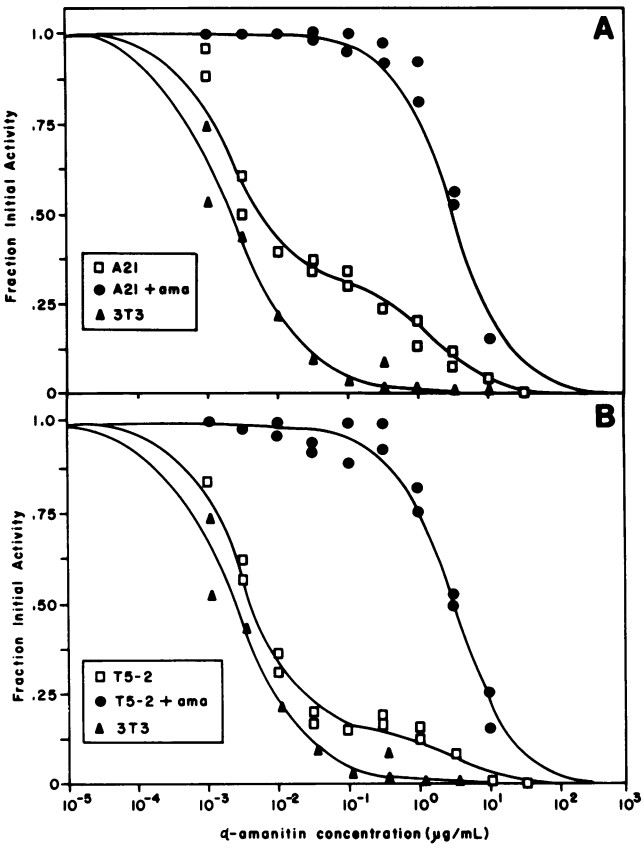

FIG. 1. Comparison of the α-amanitin sensitivity of RNA polymerase II from cell line A21 and transfectant line T5-2 with that from BALB/c 3T3 cells. RNA polymerase II activity in lysed cells was measured as described in Materials and Methods. Assays were performed in increasing α-amanitin concentrations. The incorporated counts in 34 μg of α-amanitin per ml (representing the contribution of RNA polymerases I and III) were subtracted from each point, and the value was plotted as a fraction of counts incorporated in the absence of α-amanitin. The curves fit to the experimental points were drawn by computer with the equation: fraction initial activity = $(X/1 + A/K_s) + [(1 - X)/1 + A/K_r]$, originally described by Lobban et al. (37), where A is the concentration of α-amanitin, K_s is the K_i (K_i = concentration of α-amanitin necessary for 50% inhibition) of the sensitive enzyme, K_r is the K_i of the resistant enzyme, and X is the fraction of total enzyme that is amanitin sensitive. The curves shown were fit by inspection of families of curves in which the values of K_s, K_r, and X were varied. (A) RNA polymerase II from α-amanitin-resistant cell line A21 grown in the presence or absence of α-amanitin (ama) is compared with parental BALB/c 3T3 cells. The inhibition constants of the monophasic curves for parental 3T3 and A21 plus α-amanitin are K_s = 0.008 μg/ml and K_r = 3 μg/ml, respectively. The values for the biphasic curve fit to the experimental points for A21 grown in the absence of α-amanitin are K_s = 0.002 μg/ml and K_r = 1.5 μg/ml, with X = 0.68. (B) RNA polymerase II from transfectant cell line T5-2 grown in the presence or absence of α-amanitin (ama) is compared with BALB/c 3T3. The inhibition constants of the monophasic curves for 3T3 and T5-2 plus α-amanitin are K_s = 0.008 μg/ml and K_r = 3.2 μg/ml, respectively. The values for the biphasic curve fit to the experimental points for T5-2 grown in the absence of α-amanitin are K_s = 0.003 μg/ml and K_r = 3.0 μg/ml, with X = 0.85.

The transfection experiments were repeated with mouse BALB/c 3T3 cells and mouse Ltk$^-$ cells. Again, the 3′ clone

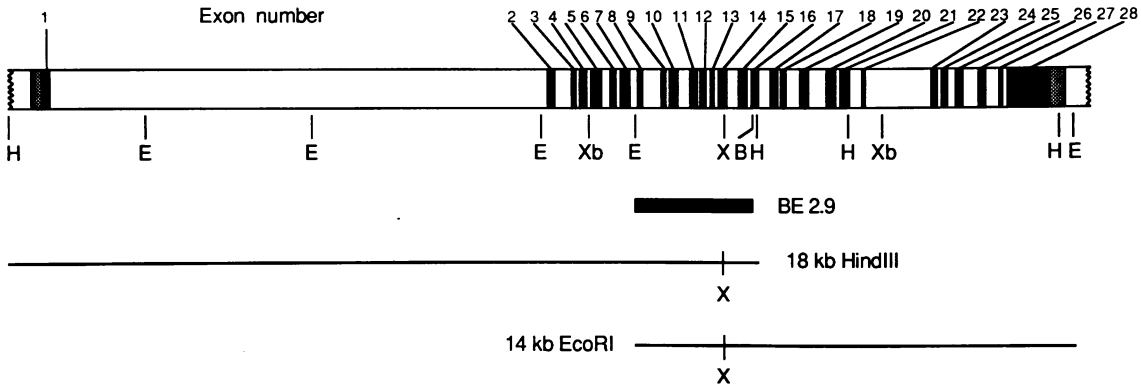

FIG. 2. Exon map of the largest mouse genomic RNA polymerase II subunit. A 30-kb segment of mouse genomic DNA is shown. The black areas are coding regions of exons (numbered above). Shaded areas are untranslated 5′ and 3′ ends, and open areas are introns. Restriction sites for *Hin*dIII (H), *Eco*RI (E), *Xho*I (X), *Xba*I (Xb), and *Bam*HI (B) are shown below the exon map (only one of several *Bam*HI sites is shown). The entire gene was cloned in the two overlapping fragments designated below the figure: the 5′ 18-kb fragment and 3′ 14-kb fragment. The probe used to isolate the fragments was designated BE2.9.

pE26-7 transferred α-amanitin resistance to both these cell lines (results not shown). L6 rat myoblasts and mouse Ltk$^-$ cells had a fivefold-higher transfection efficiency than mouse BALB/c 3T3 cells. Because L6 rat myoblasts also had a lower background of spontaneous α-amanitin resistance, they were used in most experiments.

Analysis of colonies derived from transfections with pE26-7. To verify that the amanitin-resistant RNA polymerase in the colonies obtained from transfection experiments was encoded by the A21 allele, several α-amanitin-resistant colonies derived from transfections of L6 rat myoblasts and BALB/c 3T3 cells with pE26-7 were isolated and expanded. Genomic DNA was isolated, digested with *Xba*I, and examined by Southern (52) analysis (Fig. 3). *Xba*I digestion of the endogenous *RPII215* gene from BALB/c 3T3 cells and a correctly reconstructed gene resulted in hybridization to an 8.4-kb fragment. Although nonproductively ligated and unligated fragments also hybridized with the probe, they were distinguishable from the 8.4-kb fragment by size. Lane 4 in Fig. 3 shows the 8.4-kb fragment from 3T3 cells, which is present in 1 copy per haploid genome (43). All colonies from 3T3 cell transfections will have the 8.4-kb band, but the intensity of this band will depend on the number of additional copies of *RPII215* gene the cell has incorporated into its genome (e.g., lanes 5 and 6). Because rat myoblasts do not have an endogenous 8.4-kb *Xba*I fragment (lane 1), the 8.4-kb hybridizing fragment must have arisen from correctly reconstructed DNA introduced by transfection (lanes 2 and 3). The results from the genomic blot show that the α-amanitin-resistant colonies derived from transfection contained bands corresponding to a correctly reconstructed *RPII215* gene.

To demonstrate that the α-amanitin-resistant RNA poly-

TABLE 1. Transfection of L6 rat myoblasts with normal and α-amanitin-resistant clones[a]

5′ clone	3′ clone	No. of resistant colonies/dish[b]	
		α-Amanitin	G418
pHRPII (3T3)	pEXRPII3′ (WT)	0, 0, 0	77, 83, 80
pH19-4 (A21)	λGA$_1$ (WT)	0, 0, 0	50, 53, 47
pH22-1 (A21)	pEE14.3 (WT)	1, 0, 2	47, 45, 48
pHRPII (3T3)	pE26-7 (A21)	47, 44, 42	81, 90, 83
pH19-4 (A21)	pE26-7 (A21)	48, 45, 50	64, 60, 63
pH22-1 (A21)	pE26-7 (A21)	47, 49, 43	57, 50, 49
pH22-1 (A21)	pE26-4 (A21)	1, 0, 0	47, 49, 47

[a] Three micrograms of ligated 5′ and 3′ clones were added to two 6-cm dishes by the $CaPO_4$ procedure (see Materials and Methods). After 24 h the two dishes were split into six dishes, and the following day selection with G418 and α-amanitin was started. The source of the plasmid or phage is shown in parentheses. Wild-type (WT) DNA was derived from a BALB/c 3T3 cell library, and A21 was derived from a library made from α-amanitin-resistant cell line A21.

[b] Number of α-amanitin- or G418-resistant colonies per 6-cm dish.

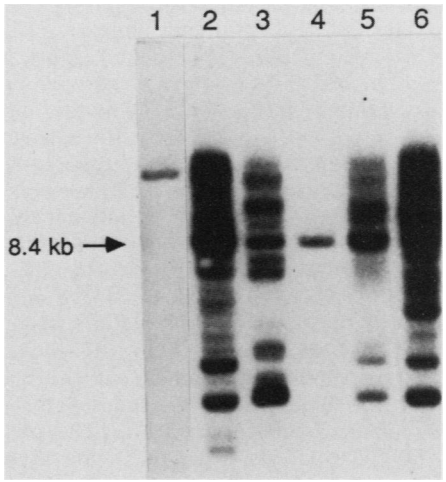

FIG. 3. Southern hybridization of *Xba*I-digested genomic DNA from L6 rat myoblast cells, BALB/c 3T3 cells, and α-amanitin-resistant transfectants. Ten micrograms of genomic DNA was digested with *Xba*I, fractionated by electrophoresis on a 1% agarose gel, and transferred to nitrocellulose. The filter was hybridized to the nick-translated probe BE2.9 for 46 h at 68°C in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate). The filter was washed for 15 min at room temperature in 2× SSC–0.5% SDS and for 1 h at 68°C in .1× SSC–0.5% SDS. Lanes 1 and 4 have DNA samples from untransfected L6 rat myoblasts and BALB/c 3T3 cells, respectively. The other lanes contain DNA samples from cells transfected with reconstructed DNA in which the α-amanitin resistance-conferring plasmid pE26-7 was used as the 3′ fragment. T6-1 (lane 2) and T6-2 (lane 3) are rat myoblast-derived transfectants, and T5-1 (lane 5) and T5-2 (lane 6) are BALB/c 3T3-derived transfectants. The arrow points to the 8.4-kb *Xba*I fragment derived from the endogenous 3T3 *RPII215* gene and correctly reconstructed transfected DNA.

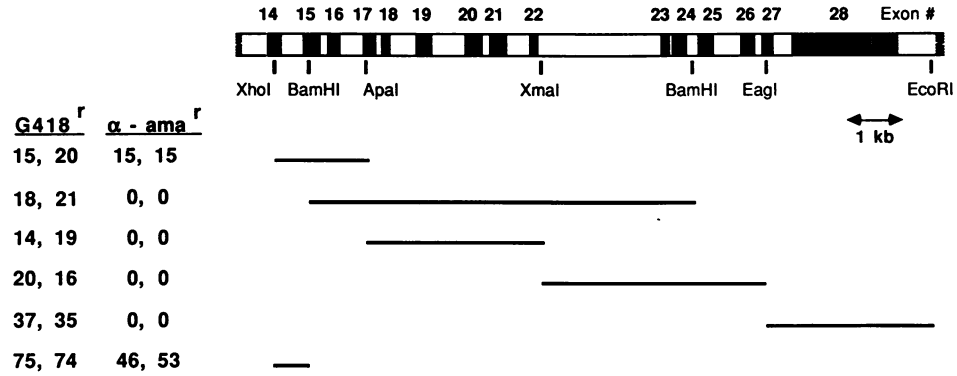

FIG. 4. Localization of α-amanitin resistance mutation. Top: Exon structure of the 3′ portion of the *RPII215* gene. The black areas are coding regions (exons numbered above), the shaded area is the untranslated 3′ end, and the open areas are introns. The *Xho*I-*Eco*RI fragment from the α-amanitin-resistant A21-derived plasmid pE26-7 transferred amanitin resistance to transfected cells. Fragments from pE26-7 were isolated (with the restriction enzymes indicated beneath the exon map) and substituted for the analogous fragments in the wild-type 3′ clone pEXRPII3′. The lines under the exon map correspond to the pE26-7-derived fragment in the resulting hybrid 3′ plasmids. Reconstructed large subunit DNA was prepared as described in Materials and Methods. Five micrograms of test DNA and 1 μg of pSV2neo were used to transfect rat myoblasts. After 24 h duplicate dishes were split into a total of six dishes, and after 48 h selection with G418 (300 μg/ml) or α-amanitin (gradually increased from 0.5 to 2 μg/ml) was started. Colonies were fixed and Giemsa stained after 3 weeks. Numbers to the left of each line indicate the number of α-amanitin- or G418-resistant colonies per 6-cm dish. In this assay the *Xho*I-*Apa*I and *Xho*I-*Bam*HI fragments from pE26-7 conferred α-amanitin resistance.

merase II produced in cells isolated from transfection experiments had the properties of the α-amanitin-resistant enzyme from the parent line A21, polymerase sensitivity was assayed in the BALB/c 3T3 transfectant T5-2 (Fig. 1B). From the curves in Fig. 1, the K_r values (the concentration of α-amanitin that inhibited polymerase activity by 50%) could be estimated. The parent line A21 (K_r = 1.5) and the transfectant line T5-2 (K_r = 3.0) had similar K_rs (the twofold difference in K_r was within experimental error). The polymerase sensitivity assay was also performed on another pE26-7 transfectant (T5-4, not shown), and this mutant had a K_r (2.5) close to that of the parent line. Because our original mutant cell lines varied over a 100-fold range in the level of resistance, the similarity in the level of resistance of the transfectant polymerases and the A21 polymerase is consistent with the idea that the gene which transfers α-amanitin resistance is derived from the resistant A21 allele.

Mapping of the A21 α-amanitin resistance mutation. To localize the mutation causing α-amanitin resistance, fragments from the A21-derived 3′ clone pE26-7 were substituted for the corresponding fragments in the wild-type 3′ clone pEXRPII3′. The hybrid 3′ clones were ligated to α-amanitin-sensitive 5′ plasmids, and this DNA was used to transfect L6 rat myoblasts (Fig. 4). In the first series of transfections (top five clones) only the *Xho*I-*Apa*I fragment from pE26-7 conferred resistance. The overlapping *Bam*HI-*Bam*HI fragment did not transfer resistance, indicating that the α-amanitin resistance mutation was located between the *Bam*HI and *Xho*I sites. This was verified by demonstrating that the *Xho*I-*Bam*HI fragment transferred resistance, thus localizing the mutation to a region containing parts of exons 14 and 15.

Identification of the mutation conferring α-amanitin resistance. The 450-base-pair *Bam*HI-*Xho*I fragment from pE26-7 was subcloned into M13mp19, and both strands were sequenced by the dideoxy method of Sanger et al. (48). A single-nucleotide change was detected in exon 15 when the sequence from pE26-7 was compared with the wild-type sequence (Fig. 5). An adenine (base number 6819 in the genomic sequence submitted to the Los Alamos nucleotide sequence library) in the wild-type coding sequence has been changed to a guanine in the mutant. This corresponds to an asparagine (residue 793)-to-aspartate change in the predicted protein sequence.

Conservation of sequences around the A21 mutation. The subunit structure of RNA polymerases from a wide variety of eucaryotic organisms shows a high degree of conservation (34, 41). Immunological studies have shown that the largest

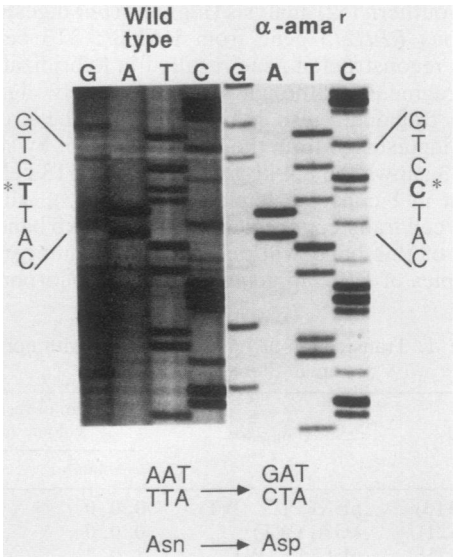

FIG. 5. Sequence surrounding α-amanitin resistance (α-amaʳ) mutation. The BE2.9 fragment from the wild-type *RPII215* gene (left) and the *Bam*HI-*Xho*I fragment from pE26-7 (right) were cloned into M13, and the noncoding strand was sequenced by the dideoxy sequencing method (48). The dideoxy termination reaction mixtures for ddGTP (G), ddATP (A), ddTTP (T), and ddCTP (C) were electrophoresed on an 8% acrylamide–urea gel. The asterisks refer to the one residue in the 450-base-pair *Xho*I-*Bam*HI fragment that varies between the wild-type and α-amanitin-resistant genes. A thymine in the wild type has been changed to a cytosine in the mutant. This corresponds to an asparagine (residue 793)-to-aspartate change in the protein, as indicated at the bottom.

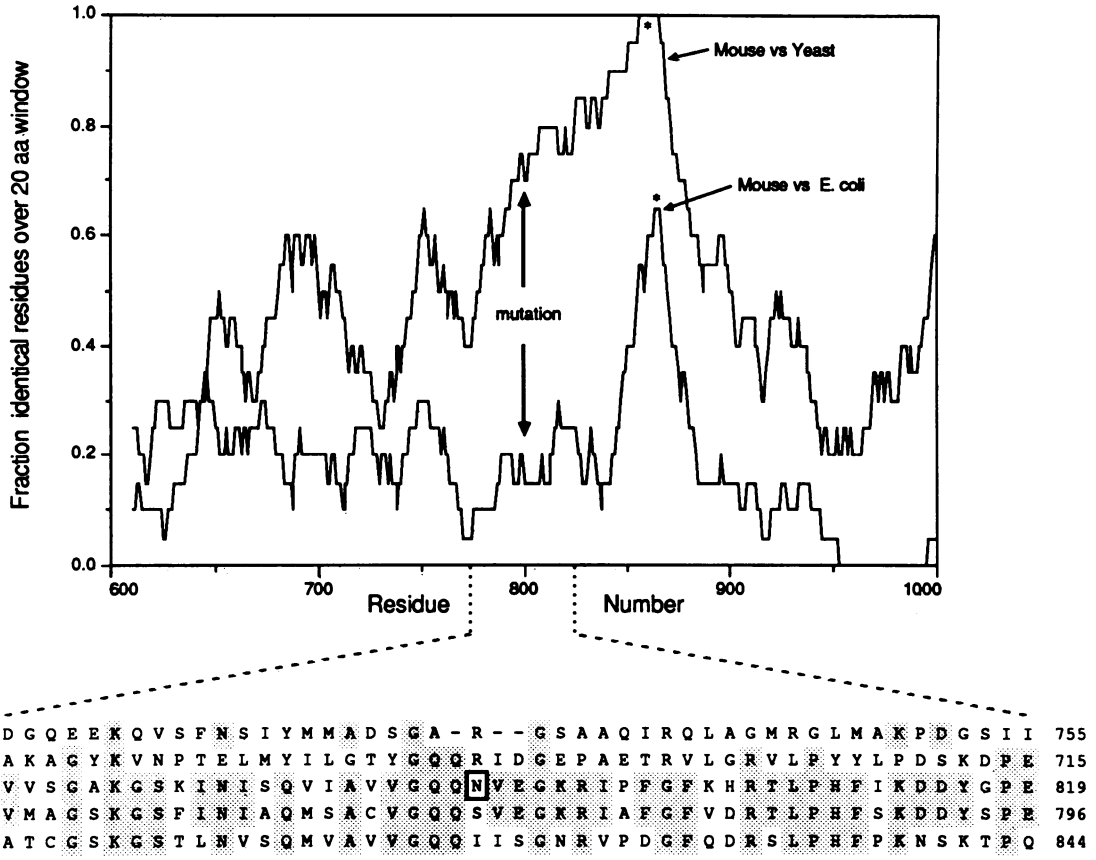

FIG. 6. Homology among RNA polymerase subunits in the region surrounding the A21 α-amanitin resistance point mutation. The homology plots shown were constructed by aligning the pairs of sequences with the computer program SEQHP (21). This program introduces gaps into each sequence to maximize homology. The aligned sequences, including gaps, were compared with the program PROT (D. P. Carbone, unpublished data), which calculates the fraction of identical residues within a window (in this case, 20 amino acids) that is moved, one residue at a time, through the sequences. The graph shows the fraction of identical residues plotted as a function of the 10th residue of the 20-amino-acid window. The position of the A21 mutation is marked by arrows on the graph. Asterisks denote a region of strong homology among procaryotic and eucaryotic subunits. The sequences compared in the graph are shown below (MII, mouse *RPII215* subunit; YII, yeast RNA polymerase II largest subunit; E.C., *E. coli* β′ subunit) together with the sequences of vaccinia virus RNA polymerase largest subunit (V) and the yeast RNA polymerase III largest subunit (YIII). The asparagine residue that is changed to aspartate in A21 is boxed. Numbers on the sequences at the bottom of the figure are from the published literature (see text for references) and do not correspond exactly to the graph above because of the introduction of gaps into each sequence. Amino acids that are identical to the mouse sequence are shaded.

subunits of the three classes of eucaryotic RNA polymerase are related (8, 28). Furthermore, within each class of RNA polymerase, the largest subunits are highly conserved among species (9, 28, 57). A particularly good example of cross-species conservation among a class of eucaryotic RNA polymerases is the α-amanitin inhibition of RNA polymerase II from a wide variety of species.

Sequence analysis of the largest subunit of RNA polymerase II from *S. cerevisiae* (1), *Drosophila* (3), and mouse (Ahearn et al., submitted) has shown that these subunits are related to each other and to the β′ subunit of *E. coli* RNA polymerase (40). The largest subunits of yeast RNA polymerase III (1) and vaccinia virus RNA polymerase (6) also belong to this family of β′-like subunits. These results suggest that the eucaryotic RNA polymerases descended from a primordial bacterial-type enzyme and that the different polymerases arose by gene duplication.

Figure 6 shows a graph of homology in the region surrounding the mutation to amanitin resistance. The sequence surrounding the mutation (boxed) is shown below the graph. It is clear from the two curves that the mutation lies in a region that is conserved between the two eucaryotic subunits, but is not conserved between the mouse and *E. coli* subunits. The mutation lies just 5′ to one of seven regions of strong homology between procaryotic and eucaryotic subunits (marked by asterisks).

In the immediate vicinity of the point mutation, the most highly conserved sequences (shaded below graph) are those of mouse and yeast RNA polymerase II. These are the only two polymerases shown in Fig. 6 that were inhibited by α-amanitin. The mouse and yeast subunits differ at the site of mutation in what otherwise would be a string of 11 consecutive identical residues. Yeast RNA polymerase II is 500 times less sensitive to α-amanitin than the mouse enzyme (55), and it is possible that this single difference (asparagine in mouse, serine in yeast) is the cause of the greater resistance of the yeast enzyme.

DISCUSSION

The amanitin resistance gene described in this paper will be a powerful tool in the study of RNA polymerase II

structure and function. First, the study of this mutation and other α-amanitin resistance mutations will elucidate the mechanism by which α-amanitin binds and inhibits RNA polymerase. Second, amanitin resistance genes can be transferred to different cell types to study potential development- or differentiation-specific phenotypes. Third, new mutations can be constructed in amanitin resistance genes to search for novel phenotypes.

Amanitin binding and transcription elongation. Labeled derivatives of α-amanitin bind tightly to RNA polymerase with an equilibrium association constant of 10^8 to 10^{10} M^{-1} (13). Ingles et al. (29) characterized a series of α-amanitin-resistant Chinese hamster ovary cell lines containing RNA polymerases 5 to 500 times more resistant to the toxin than is the wild-type enzyme. The altered sensitivity of these mutant enzymes is proportional to their reduced ability to bind α-amanitin (29). Mutant RNA polymerase II from *Drosophila* (15) and human cell lines (50) also show reduced ability to bind α-amanitin. Although we have not yet measured the binding of α-amanitin to our mutant enzyme, it seems likely that the basis of resistance is decreased binding.

What does the A21 mutation tell us about the structure and function of RNA polymerase II? Although the precise mechanism is not known, amanitin seems to block elongating polymerase by binding to the enzyme and inhibiting translocation of the template-primer complex after phosphodiester bond formation (54). Coulter and Greenleaf (16) have shown that an amanitin-resistant *Drosophila* RNA polymerase II elongates nascent RNA at half the rate of the wild-type enzyme, indicating that a mutation that affects amanitin binding can also affect elongation of RNA polymerase II. It is possible that the region surrounding the A21 mutation is involved not only in binding amanitin but also in the process of transcription elongation. Characterization of other amanitin-resistant genes together with detailed amanitin cross-linking studies will be necessary to define the amanitin-binding site. Analysis of the region surrounding the A21 mutation by localized random mutagenesis may help to identify other residues involved in amanitin binding or transcription elongation.

Developmental phenotypes of amanitin-resistant RNA polymerase II. Resistance to α-amanitin also results in interesting developmental phenotypes. In *Drosophila* the amanitin-resistant mutation *C4* can enhance the effects of the homeotic mutation *Ubx* (24). Furthermore, *C4* is allelic with another mutation (*Ubl*) that can interact with a number of developmentally regulated loci (39). An intriguing possibility is that the promoter selectivity of these mutant polymerases is altered (24).

Mutant rat myoblast cell lines that have been selected for resistance to α-amanitin also show interesting phenotypes. About one-third of these cell lines display an amanitin-dependent myogenic differentiation-defective phenotype (18). This phenotype is presumably caused by inactivation of the wild-type enzyme in the presence of amanitin and failure of the resistant enzyme to carry out the transcriptional program necessary for myogenic differentiation. In the experiments described in this paper we have used the same (L6) rat myoblast cell line as Crerar et al. (18). All of the amanitin-resistant colonies resulting from transfection of A21 DNA were able to form myotubes in culture (as monitored by light microscopy; unpublished data). Thus, the mouse amanitin-resistant enzyme described in our study does not seem to be altered in its ability to support myogenic differentiation in rat cells. Whether the A21 mutant enzyme is altered in its ability to support other developmental or differentiation pathways can be tested by transfecting the mutant gene into the appropriate cell lines or by constructing transgenic mice containing the mutant *RPII215* allele.

New mutations. The A21 mutation will also be valuable for introducing genetically altered *RPII215* genes into cells. The A21 gene can be further altered by using in vitro mutagenesis, and the effects of such changes can be monitored by transfection. Lethal mutations will fail to transfer amanitin resistance, while nonlethal mutations can be screened for the conferral of novel phenotypes. We are currently using this approach to study the effect of deleting all or parts of the repeated C-terminal domain (14) of the mouse *RPII215* subunit.

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